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A Multiplex RT-PCR Test for the Differential Identification of Turkey Astrovirus Type 1, Turkey Astrovirus Type 2, Chicken Astrovirus, Avian Nephritis Virus, and Avian Rotavirus

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SUMMARY. Recent studies have revealed the presence of astroviruses and rotavirus in numerous poorly performing and healthy chicken and turkey flocks in the United States. The phylogenetic analysis of the sequence data produced during these studies has identified four groups of avian astroviruses circulating in the United States: turkey astrovirus types 1 and 2 (TAstV-1 and TAstV-2), avian nephritis virus (ANV), and a chicken-origin astrovirus (CAstV). As the molecular epidemiology of poultry enteric disease is poorly understood, the development of updated diagnostic assays is crucial to the continued surveillance and management of enteric disease in affected as well as healthy flocks. This report details the development of a multiplex reverse transcriptase–polymerase chain reaction (RT-PCR) assay specific for astroviruses and avian rotavirus in turkey-origin and chicken-origin samples. The assay consists of two multiplex tests, one for turkey-origin samples and one for chicken-origin samples. The turkey sample test differentially identifies TAstV-1, TAstV-2, ANV, and avian rotavirus. The test for chicken-origin samples differentially identifies CAstV, ANV, and avian rotavirus. Assay sensitivity varied by target sequence between approximately 10 copies for avian rotavirus alone and approximately 2×10^6 copies for TAstV-2 in the presence of a heterologous competitor RNA sequence. Each test was shown to be specific for the intended target by testing for cross-reaction with other common avian enteric viruses. The specificity was further shown by testing 109 chicken specimens and 32 turkey specimens from commercial flocks with the appropriate test and sequencing the RT-PCR amplicons to confirm amplification of the correct target.

RESUMEN. Prueba múltiple de RT-PCR para la identificación diferencial de Astrovirus tipo 1 del pavo, astrovirus del pavo tipo 2, astrovirus del pollo, virus de nefritis aviar y rotavirus aviar.

Estudios recientes en los Estados Unidos han revelado la presencia de astrovirus y rotavirus en numerosos casos de lotes de pollos y pavos sanos con mal desempeño. El análisis filogenético de la secuencia de datos producida durante estos estudios ha identificado cuatro grupos de astrovirus aviares circulando en los Estados Unidos: Astrovirus del pavo tipo 1 y 2 , virus de la nefritis aviar y astrovirus originados en pollos. Debido a que la epidemiología molecular de las enfermedades entéricas en aves domésticas no está bien entendida, el desarrollo de pruebas de diagnóstico actualizadas es crucial para continuar la vigilancia y el manejo de la enfermedad entérica tanto en lotes afectados como en lotes sanos. Este reporte detalla el desarrollo de una prueba múltiple de reacción en cadena por la polimerasa - transcriptasa reversa específica para astrovirus y rotavirus aviares en muestras de pavos y pollos. El ensayo consiste en dos pruebas múltiples, una para muestras originadas en pavos y otra para muestras originadas en pollos. La prueba para muestras de pavo identifica diferencialmente Astrovirus del pavo tipo 1 y 2, virus de la nefritis aviar y rotavirus aviar. La prueba para muestras originadas en pollos diferencia astrovirus de pollos, virus de la nefritis aviar y rotavirus aviar. Los ensayos de sensibilidad variaron la secuencia blanco únicamente entre aproximadamente 10 copias para rotavirus aviar y aproximadamente 2×10^6 copias de Astrovirus del pavo tipo 2 en la presencia de un competidor heterólogo en la secuencia de RNA. Cada prueba mostró ser específica para el objetivo planeado mediante la realización de pruebas de reacciones cruzadas con otros virus entéricos aviares comunes. La especificidad de la prueba fue ampliamente demostrada probando 109 muestras de pollo y 32 de pavo de lotes comerciales con la prueba adecuada y secuenciando los amplicones resultantes de la prueba RT-PCR para confirmar la amplificación del objetivo correcto.

Key words: astrovirus, avian nephritis virus, enteric viruses, multiplex RT-PCR

Abbreviations: ANV = avian nephritis virus; CAstV = chicken-origin astrovirus; cDNA = complementary DNA; HEV = hemorrhagic enteritis virus; LOD = limit of detection; RT-PCR = reverse transcriptase–polymerase chain reaction; TAstV = turkey-origin astrovirus

Among the numerous viruses commonly identified in the intestinal contents of chickens and turkeys with enteric disease are the four types of avian astroviruses and avian rotavirus (1,3,5,7,10,12,13,14,15,17,18,19,25). Although reverse transcriptase–polymerase chain reaction (RT-PCR) tests for avian astroviruses are available (8,9,21,22,23,24), they are either broadly reactive or detect viruses from all types; therefore, it is necessary to sequence the RT-PCR product to determine which astrovirus type is present (15). In addition, with this format, concomitant infection with multiple astrovirus types would be missed. Conversely, some avian astrovirus RT-PCR tests only detect a limited number of strains within a type, since they were developed with sequence information from only a limited number of virus strains (9). Since sequence information for both avian astroviruses (6,14,15,24) and rotavirus has increased recently, improved diagnostic tests can be developed. Chickens and turkeys can each be infected with numerous types of astrovirus and with rotavirus, and frequently these infections are concomitant (14,15); therefore, differential identification of astrovirus types is critical to elucidating the role of each astrovirus type in disease.

In this report we describe the development of two differential, multiplex, conventional RT-PCR tests for avian astroviruses based on sample species of origin. As a result of its prevalence in poultry intestinal samples, avian rotavirus was also included in the test to improve the efficiency of testing for enteric viruses. The multiplex RT-PCR test for chicken-origin specimens targets chicken astrovirus

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Table 1. Primer sequences used in multiplex RT-PCR (IUB codes used: Y = pyrimidine, R = purine, K = G or T, M = A or C).

Target virus	Target gene	Primer name	Primer sequences
TAstV-1	Polymerase	T1 pol 1F	5′-AGC TYA TGM GGT TCT TTC TTC TYG-3′
		T1 pol 1R	5′-GAT GGT GGG TAG CCT ATT GTG TTC-3′
TAstV-2	Polymerase	T2 pol 2F	5′-TGG ACC GAC CCR RTT TTY ACC A-3′
		T2 pol 2R	5′-GGC CCG ACY TCA GGM AGT TGT-3′
CAstV	Polymerase	CAS pol 1F	5′-GAY CAR CGA ATG CGR AGR TTG-3′
		CAS pol 1R	5′-TCA GTG GAA GTG GGK ART CTA C-3′
ANV	Polymerase	ANV pol 1F	5′-GYT GGG CGC YTC YTT TGA YAC-3′
		ANV pol 1R	5′-CRT TTG CCC KRT ART CTT TRT-3′
Avian rotavirus	NSP4	NSP4 F30	5′-GGG CGT GCG GAA AGA TGG AGA AC-3′
		NSP4 R660	5′-GGG GTT GGG GTA CCA GGG ATT AA-3′

(CAstV), avian nephritis virus (ANV), and avian rotavirus. The multiplex RT-PCR test for turkey-origin samples targets turkey astrovirus (TAstV) types 1 and 2, ANV, and avian rotavirus.

MATERIALS AND METHODS

Multiplex RT-PCR test. The primer sequences designed for use in this study are listed in Table 1. The turkey-specific test contained primer pairs that targeted the polymerase gene (ORF 1B) of TAstV-1, TAstV-2, and ANV and the NSP4 gene from avian rotavirus. The chicken-specific test contained primer pairs that targeted the polymerase gene (ORF 1B) from CAstV and ANV and the same avian rotavirus NSP4 gene primers used in the turkey sample test. Primer pairs were designed to produce amplicons easily distinguished by agarose gel electrophoresis based upon size (Fig. 1).

Conventional RT-PCR using RNA isolated from field samples and *in vitro*–transcribed RNA was performed using the Qiagen One-Step RT-PCR kit (Qiagen, Inc., Valencia, CA). Each 25-μl reaction contained 1× Qiagen reaction buffer, 320 μM of each dNTP, 0.8 μM of each primer, 1 μl of Qiagen enzyme blend, and 2.5 μl of extracted RNA. The absolute amount of RNA added to each reaction varied based upon the origin of the sample and upon the dilution factor of the *in vitro*–transcribed RNA. Amplification was performed in a MJ Research DNA thermocycler (MJ Research, Waltham, MA). Thermal cycling consisted of one cycle of 50 C for 30 min, one cycle of 94 C for 15 min, followed by 35 cycles of 94 C for 30 sec, 55 C for 30 sec, and 72 C for 1 min. The amplicons were separated by standard agarose gel electrophoresis.

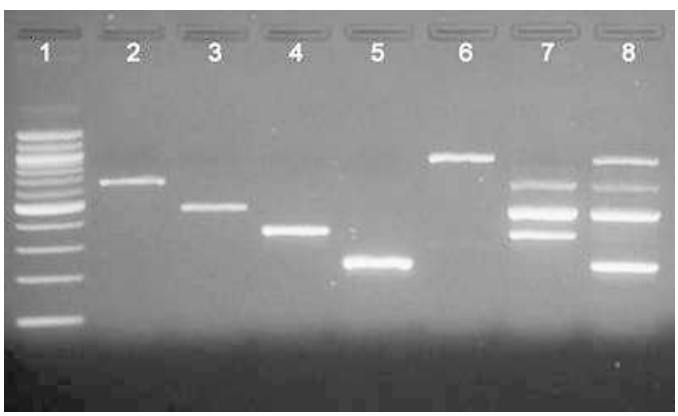

Fig. 1. Agarose gel (1.2%) with RT-PCR amplicons generated from *in vitro*–transcribed RNA for the five individual primer pairs in the multiplex test. Lane 1: 100–base pair (bp) ladder; Lane 2: avian rotavirus (630 bp); Lane 3: ANV (473 bp); Lane 4: CAstV (362 bp); Lane 5: TAstV-1 (251 bp); Lane 6: TAstv-2 (911 bp); Lane 7: all three target amplicons included in the chicken-origin test; Lane 8: all four target amplicons included in the turkey-origin test.

Determination of assay limits of detection with *in vitro*–transcribed RNA. Assay sensitivity was determined for each target alone and in the presence of at least one other target to evaluate the effect of concomitant infection on sensitivity. RNA was produced by *in vitro* transcription of a complementary DNA (cDNA) template of the target gene that was larger than the target RT-PCR amplicon. The T7 transcription template was produced by standard RT-PCR of a viral gene target sequence using primers that lie outside the multiplex RT-PCR target and that contain the T7 promoter sequence at their 5′ ends. The *in vitro* transcriptions were performed with the Promega T7 Ribomax Express Large Scale RNA Production System (Promega, Madison, WI), according to the manufacturer's recommendations. After the *in vitro* transcription, the RNA was purified from the template by treatment with DNase and subsequent extraction with TRIzol LS reagent (Invitrogen, Inc., Carlsbad, CA) and was reconstituted in RNase-free water. Each template was confirmed to be cDNA free by running a sample of the *in vitro*–transcribed RNA preparation in a PCR reaction; if a reaction product was present, the DNase treatment and TRIzol steps were repeated.

The concentration of each *in vitro*–transcribed RNA standard was determined using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). The minimum amount of RNA that each test could detect was determined by performing a 10-fold dilution series of each *in vitro*–transcribed RNA template and performing the RT-PCR assays, as described, for each virus target singly and in a separate reaction with the addition of a heterologous competitor RNA (*in vitro*–transcribed RNA from another virus targeted by the reaction) to mimic the presence of multiple viral targets in a clinical sample. The gene copy limit of detection (LOD) was determined for each RNA both in a single reaction and in combination with the heterologous competitor. RNA target competitors were selected based on which are seen most frequently as concomitant infections in the field: ANV for avian rotavirus, CAstV for ANV, TAstV-2 for TAstV-1, TAstV-1 for TAstV-2, and ANV for CAstV. Competitors were added at a constant concentration (the $\log_{10}$ dilution above the LOD) for each dilution series.

Evaluation of assay specificity by sequencing amplicons from field samples. In 2005, intestinal contents were collected from commercial turkey and chicken flocks from several regions of the United States. The samples were collected from both healthy and poorly performing flocks. Chickens were sampled between 5 days and 14 days of age, and turkeys were sampled between 5 days and 84 days of age. All samples were stored at −80 C until examined and utilized for RNA extraction.

RNA was extracted by diluting intestinal contents (200 μl) in 1.2 ml of cold phosphate-buffered saline in sterile conical tubes that were then shaken by hand three times for 30 sec each time. The tubes were then centrifuged at 3000 × *g* (at 4 C) for 10 min. The supernatant was placed in a fresh tube and stored at −80 C until use. Total RNA was extracted directly from 250 μl of the supernatant using TRIzol LS reagent (Invitrogen), according to the manufacturer's recommendations, and was reconstituted in 100 μl of 90% dimethyl sulfoxide. The RT-PCR and gel electrophoresis were performed as described above.

Table 2. Approximated limits of detection (LODs).

Test specificity	LOD Single target	LOD Target + competitor
TAstV-1	1.33×10^{4}[A]	1.33×10^{4}
TAstV-2	1.98×10^{5}	1.98×10^{6}
CAstV	6.17×10^{2}	6.17×10^{2}
ANV	4.22×10^{5}	4.22×10^{5}
Rotavirus	10	10

[A]RNA target copy number.

Amplicons of the expected size for each test were selected and gel extracted using the QIAquick gel extraction kit (Qiagen). Targets were sequenced with the same primers used in the RT-PCR reactions, which corresponded to the expected target, based on fragment size, using the BigDye terminator kit (Applied Biosystems, Foster City, CA) and an AB 3730 DNA sequencer (Applied Biosystems).

Of the total chicken samples ($n = 109$) that were subjected to the chicken-origin multiplex RT-PCR test, 16 of 37 positive avian rotavirus–specific, 20 of 61 positive ANV-specific, and 23 of 52 positive CastV-specific amplicons were selected for DNA sequencing. Of the total turkey samples ($n = 32$) that were subjected to the turkey-origin multiplex RT-PCR test, 13 of 23 positive TAstV-2–specific, 4 of 10 positive avian rotavirus–specific, 5 of 7 positive ANV-specific, and 14 of 17 positive TAstV-1–specific amplicons were selected for DNA sequencing. In total, 20 avian rotavirus, 25 ANV, 23 CAstV, 14 TAstV-1, and 13 TAstV-2 amplicons were sequenced. The resulting DNA sequences were subjected to BLASTn searches to confirm the identity of the product.

Evaluation of cross-reaction with other common avian viruses that may be present in intestinal samples. RNA or DNA from selected common avian enteric viruses (turkey coronavirus, chicken-origin avian reovirus [S1133 isolate], turkey-origin reovirus [NC/SEP-R44/03 isolate], and hemorrhagic enteritis virus [HEV]) and type 1 avian adenovirus were tested with each multiplex test under the optimized conditions.

RESULTS

Assay sensitivity. Limits of detection were determined using *in vitro*–transcribed RNA standards for each viral gene target. In single template reactions, limits ranged from approximately 10 RNA copies for the rotavirus primer pair to 4.22×10^{5} RNA copies for the ANV-specific primer pair and from 10 copies for rotavirus to 1.98×10^{6} for TAstV-2 in the presence of a heterologous competitor RNA (Table 2). The presence of a competitor template only affected the assay limit of detection with the TAstV-2–specific primers, which showed a weak signal (band) with 1.98×10^{5} gene copies in the single reaction. This weak band disappeared when the heterologous competitor (TAstv-2 RNA) was present.

Specificity with field samples. All amplicons that were submitted for sequence analysis were confirmed to be the expected target. A total of 20 samples that were expected to be avian rotavirus based on the multiplex test were confirmed to be the correct target by sequencing (Table 3). Twenty-five ANV, 23 CAstV, 14 TAstV-1, and 13 TAstV-2 samples that were expected to be those respective astrovirus types were all confirmed by comparison to previously sequenced avian astrovirus isolates (15) and subsequent BLASTn analysis (Table 3).

Cross-reaction with other avian enteric viruses. Neither multiplex test produced any products with RNA or DNA from the avian reovirus strains NC/SEP-R44/03 and S1133, turkey coronavirus, HEV, or type 1 adenovirus.

DISCUSSION

Although enteric viruses in poultry have been recognized for decades, there is still much to learn about poultry enteric disease. Recent molecular epidemiologic studies have revealed the widespread occurrence of enteric viruses in healthy and poorly performing flocks (3,14,15), and the continuing presence in the United States of multifactorial enteric disease of poultry, such as poult enteritis complex and runting–stunting syndrome of broilers, indicates that updated diagnostic and control measures are needed (1,2,4,16,20). Astroviruses are particularly widespread in U.S. chicken and turkey flocks (14,15), and several molecular-based diagnostic tests target the avian astroviruses (9,21,22,23). However, an improved diagnostic test for avian astrovirus is warranted, since multiple types of astrovirus have been found, often as concomitant infections in U.S. poultry flocks. Until now, the differentiation of these multiple types has required the sequencing of viral cDNA and subsequent typing. The multiplex RT-PCR test described in this article is designed to differentiate among the astrovirus types presently circulating in U.S. poultry: TAstV-1, TAstV-2, ANV, and CAstV. These two bench-validated tests can be used on samples of either turkey or chicken origin, and the addition to the tests of a primer pair targeting avian rotavirus has combined several molecular diagnostic tests, producing a more efficient and cheaper alternative to multiple tests followed by sequence analysis.

Because there are no detection reference standard methods for these viruses and no reliable titration methods, *in vitro* methods were used to evaluate assay sensitivity. The analytical sensitivity did vary by target during the LOD determination with *in vitro*–transcribed RNA targets, and although the LOD is higher than a previously described multiplex real-time RT-PCR test that included TAstv-2 as a target (22), this differential test is intended for use on a flock basis, where lower sensitivity is less of a concern. Further, the astroviruses tend to be shed at very high titers (11), and they should be detected even at the LOD shown for this test. It is important to note that in order to preserve the differential nature of this multiplex test, the astrovirus primer pairs are degenerate (Table 1), a fact that probably contributed to the relatively high LOD. The rotavirus portion of the test, which is not based on degenerate primers, had a much lower LOD (Table 2).

The true strength of this differential test was demonstrated when the specificity of the test was confirmed by sequencing the cDNA

Table 3. Number of multiplex RT-PCR positive samples confirmed as the expected target by gene sequencing.

Virus target	Positive chicken-origin samples ($n = 109$)	Positive turkey-origin samples ($n = 32$)	Total positive samples	Target confirmed by sequencing
TAstV-1	N/A[A]	17	17	14
TAstV-2	N/A	23	23	13
CAstV	52	N/A	52	23
ANV	61	7	68	25
Rotavirus	37	10	47	20

[A]N/A = not applicable.

amplicons produced from field samples during the use of the test for a survey of enteric viruses in the United States. The results summarized in Table 3 demonstrate that 100% of the selected amplicons were specific for the virus gene targeted by the test. Further, the results from the survey revealed numerous concomitant infections in both turkeys and chickens (i.e., with two or more astroviruses and/or avian rotavirus) that would not have been identified with earlier tests targeting avian astrovirus. Finally, although this is not a comprehensive panel for the molecular diagnosis of avian enteric viruses, the specificity of the test was further demonstrated by the lack of cross-reactivity with several other common avian enteric viruses: turkey coronavirus, two types of avian reovirus, HEV, and type 1 adenovirus.

This article represents the initial bench validation and initial field application of a differential multiplex RT-PCR test for four types of avian astrovirus and rotavirus. As more sequence data become available for the poultry enteric viruses, it should be possible to include in the test panel primers able to differentiate the avian rotavirus groups. For now, the avian rotavirus NSP4 gene is not group specific, so all rotavirus groups could likely be detected. However, sequence information for all rotavirus types is not currently available for comparison, so confirmation cannot be performed at this time. This test should prove valuable for diagnostic and research activities as the avian enteric viruses become better characterized and as the molecular epidemiology of avian enteric disease improves.

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